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Human SM22α BAC encompasses regulatory sequences for expression in vascular and visceral smooth muscles at fetal and adult stages

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Xu, Rui, Ye-Shih Ho, Raquel P. Ritchie, and Li Li. Human *SM22α* BAC encompasses regulatory sequences for expression in vascular and visceral smooth muscles at fetal and adult stages. *Am J Physiol Heart Circ Physiol* 284: H1398–H1407, 2003; 10.1152/ajpheart.00737.2002.— The *SM22α* gene has widely been used to study the regulatory mechanisms of smooth muscle cell (SMC) gene expression during cardiovascular development. To determine the regulatory mechanisms for the evolutionarily conserved human *SM22α* (h*SM22α*) gene, we demonstrated that 445 bp upstream DNA sequences of h*SM22α* gene exhibited a high transcriptional activity in arterial SMC, not in venous nor in visceral SMCs during embryogensis. However, this promoter was gradually turned off in adulthood. Inclusion of the first intron in this promoter suppressed the promoter activity in pulmonary trunk arterial SMCs, whereas the expression in other systemic vasculature remained similar to that of the h*SM22*-445 promoter during the fetal and adult stages. To determine whether additional sequences are required for *SM22α* expression in all subtypes of SMCs, we examined the expression of a bacterial artificial chromosome containing the h*SM22α* locus in transgenic mice. The h*SM22α* transgene showed similar developmental expression patterns as the endogenous mouse *SM22α* gene, suggesting that this bacterial artificial chromosome contains essential regulatory sequences for its expression in arterial, venous, and visceral tissues during development.

bacterial artificial chromosome; regulatory element; intron; pulmonary trunk

SMOOTH MUSCLE CELLS (SMCs) are generally categorized as vascular and visceral subtypes and are highly plastic and heterogeneous in origin (6). Whereas visceral SMCs develop from local mesenchymal cells, vascular SMCs have at least two origins: neural crest and mesodermal cells (5, 14). The different types of SMCs may account for their diverse functions in a variety of biological systems, including circulatory, genitourinary, respiratory, and digestive (7). To better understand the phenotypic changes of different SMCs during physiological and pathological processes, the regulatory mechanisms that control SMC gene expression have been studied extensively. In particular, several SMC-specific gene markers including *SM22α*, SM-myosin heavy chain (MHC), SMα-actin, and calponin have been used as models to delineate the transcriptional mechanisms for SMC gene regulation (11, 16, 19, 21, 24, 26).

SM22α, also called transgelin, is expressed abundantly and specifically in vascular and visceral SMCs in adults (13, 15). During development, *SM22α* is first detected in all muscle lineages at early embryonic stages; it gradually diminishes in the heart and somites as embryogenesis proceeds. The expression becomes restricted to SMCs during late embryogenesis and postnatal development (15). Structurally, the *SM22α* gene contains five exons and four introns, and the proximal 5′ upstream DNA sequences are highly conserved from chicken to human (17). Using the transgenic mouse approach, we have determined that the m*SM22* promoter, containing two critical $CC(AT)_6GG$ (CArG) boxes, is sufficient to direct gene expression in all three muscle lineages at early embryonic stages but is restricted to arterial SMCs at late stages (11, 16, 17, 26, 28). The CArG box, a consensus sequence for the serum response factor binding, plays an essential role in SMC gene regulation in vitro and in vivo (2, 3, 17, 20, 22, 23, 28). However, no additional regulatory elements have been identified in up to 2.7 kb upstream DNA sequence for expression in other types of SMCs in transgenic mice (16, 26).

Because of the highly specific expression patterns, the *SM22* promoter is often sought as a tool to deliver therapeutic genes into arterial SMCs. To validate the possibility of using the human *SM22* (h*SM22*) promoter in gene therapy, we showed that the highly homologous 445 bp 5′ upstream DNA sequence of the h*SM22* gene is conserved in gene regulation. Furthermore, we analyzed the expression patterns of the lacZ transgene driven by another h*SM22* promoter, which contains 445 bp and the intron I. This analysis revealed unexpected regulatory modules in the pulmonary trunk SMC. To determine whether additional sequences are required for *SM22* expression in all subtypes of SMCs, we characterized á h*SM22α* BAC in transgenic mouse. Our analyses showed that the expression of this BAC transgene mirrors that of endogenous *SM22α*, suggesting that the h*SM22α* BAC contains essential regulatory elements for its expression

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in different subtypes of SMC in vivo. Taken together, these results provide further evidence to support the notion that the SMCs are highly heterogeneous and SMC-specific gene expression is controlled by a combination of regulatory modules in different subtypes of SMCs.

MATERIALS AND METHODS

Construction of hSM22-445/lacZ and hSM22-445-intron I/lacZ reporters. The highly conserved h*SM22α* promoter was polymerase chain reacted with the use of a pair of primers 5′-TCCCCAGCCCCTTGCCCCTC-3′ and 5′-ACGGCGGATCCGGCTTCCTCAGGGCTCGCAG-3′, spanning the 445-bp upstream sequence and partial exon I of h*SM22α*. The PCR product was cloned between the *Not*I and *Bam*H I sites in pBSSK-AUGbGAL to control the expression of the lacZ reporter (h*SM22*-445/lacZ). Another h*SM22* promoter spanning the 445 bp h*SM22* promoter, exon I, and the first intron I was polymerase chain reacted with the use of another pair of primers (5′-TCCCCAGCCCCTTGCCCCTC-3′ and 5′-CTGGGGAAAGCTAAAGCAGGCC-3′) and was cloned into the same lacZ reporter vector (h*SM22*-445-intron I/lacZ). The sequences of both promoters were determined with the use of a sequencing kit (ABI BigDye Terminator; Wayne State University Genomic Core Facility). For microinjection to generate transgenic mice, both h*SM22*-445/lacZ and h*SM22*-445-intron I/lacZ DNA fragments were released by restriction enzyme digestion with *Not*I and *Hind*III, followed by gel purification. Transgenic mice were generated in FVB inbred embryos at Wayne State University and in B6SJL/F1 (Jackson Laboratories) embryos at the core facility of Cold Spring Harbor Laboratory.

Cell culture and transfection. Cultured rat pulmonary arterial cells (PAC1) and rat thoracic artery SMC cell line (A7r5 cells) were grown in six-well plates in DMEM containing 10% serum at 37°C with 5% CO_2. When cells were 70% confluent, transient transfection was performed using Lipofectamine-PLUS reagent following the manufacturer's instructions (Life Technologies). For each experiment, 1 μg of h*SM22*-445/lacZ, h*SM22*-445-intron I/lacZ, or BS-lacZ (a promoterless lacZ vector) was cotransfected with 0.1 μg of pSM1344-luc vector. The pSM1344-luc was used as the internal control for transfection efficiency. The expression of the reporter genes was analyzed 48 h after transfection by measuring β-galactosidase activity and luciferase activity following the manufacturer's instructions (Promega). The activity of BS-lacZ was used as a background for transfection assays in PAC1 and A7r5 cells. Relative activities of the *SM22* promoters were calculated after subtracting the basal activity of BS-lacZ. The results shown were the mean of two independent triplicate experiments. The mean activity of the h*SM22*-445/lacZ was designated as 100%.

β-Galactosidase activity analysis in transgenic mice. Transgenic embryos at different developmental stages and adult mice were collected and processed as previously reported (16). Briefly, samples were fixed in 4% paraformaldehyde/PBS for 30 min at 4°C, followed by a rinse with PBS. Then samples were stained with 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Life Technologies) solution composed of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 μg/ml X-Gal, and 0.2% Nonidet P-40 overnight at room temperature. For better visualization of the vasculature of the embryos, the stained embryos were dehydrated in 100% methanol for 2 days and cleared in a solution of 2 vols of benzyl benzoate per volume of benzyl alcohol for 10 min to 1 h before being photographed.

Screening and mapping of BAC harboring hSM22α gene. A h*SM22α* BAC was cloned from the human BAC library (Research Genetics) by using the same primers as those used for cloning the h*SM22α* promoter. In addition, the primers (5′-CAGCCCTGGCCAAGCTTTGA-3′ and 5′-GGCAGGCTGGGCTGGTTCTTC-3′) spanning the 3′ UTR region of the h*SM22α* gene were used to confirm the presence of h*SM22α* in the BAC. A pair of primers (5′-TTCCCCAGCCCCTTGCCCCTC-3′ and 5′-GGCAGGCTGGGCTGGTTCTTC-3′) spanning the h*SM22α* was also used to confirm the BAC. One BAC, named BAC012399, contained 150 kb genomic DNA fragment covering the complete h*SM22* locus and two closely linked CGI-40 and PCSK7 genes. BAC012399 was cloned into *Bam*HI and *Hind*III sites in pBeloBAC11 vector and could be linearized by *Not*I digestion according to instructions from Research Genetics.

The BAC012399 clone in HS996 cells was grown in Luria-Bertani in the presence of chloramphenicol (12.5 μg/ml). BAC DNA was isolated using the QIAGEN BAC DNA isolation kit. By restriction enzyme digestion with *Not*I and pulse-field gel electrophoresis, the size of BAC012399 was estimated to be ~150 kb. Recently, the h*SM22α* gene has been mapped to the chromosome 11q23.2 and consists of five exons. By PCR and Southern blot analysis, we confirmed that BAC012399 contained the whole sequence of human chromosome 11 cosmid cSRL16b6 (U73638).

For transgenic mouse generation, the BAC012399 DNA fragment was isolated after pulse-field gel electrophoresis with low-melting agarose, followed by gel purification using drop dialysis on the membrane against microinjection solution (http://www.med.umich.edu/tamc/BACDNA.html). To determine the orientation of the *SM22α* gene in the BAC, primers (BACendF located at one end of the vector close to the SP6 promoter, 5′-GATTACGCCAAGCTATTTAGGTGACACTAT-3′, and BACendR located at the other end of the vector close to the T7 promoter, 5′-TAATACGACTCACTATAGGGCGAATTCGAG-3′) were used to examine the sequences at both ends of the BAC DNA. Meanwhile, databases from GenBank and Celera were used to determine the neighboring gene organization in the BAC.

Sequence alignment of hSM22 and mSM22α promoter. About 20 kb sequences at the m*SM22α* and h*SM22α* loci were obtained from Celera Databases. To identify homologous regions in the regulatory regions, we did homology searches with the use of the Blast program (http://www.ncbi.nlm.nih.gov/blast).

In situ hybridization. Mouse embryos and adult tissues were collected and fixed in 4% paraformaldehyde at 4°C overnight. For in situ hybridization, 6-μm paraffin-embedded tissue sections were deparaffinized, rehydrated, and treated with 10 μg/ml of proteinase K at 37°C for 15 min. The 3′ UTR RT-PCR product of h*SM22* was cloned into *Xcm*I sites of pT-NOT, a vector for TA-PCR cloning, and the insert was released using *Not*I for subcloned into *Not*I sites of pZERO-2. The construct was linearized with the use of *Spe*I and *Xho*I, respectively, for antisense and sense riboprobe synthesis (with T7 RNA polymerase and SP6 RNA polymerase respectively). Both probes were labeled with digoxingenin (Roche Diagnostics). The hybridization protocol has been described previously (29). Briefly, hybridization with 0.5 μg/ml of probe in hybridization solution was performed at 65°C overnight in a humid incubator. The hybridization buffer consisted of 50% formamide, 2 × SSC, 5 mM EDTA, pH 8.0, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 100 μg/ml heparin. Signal development and detection were performed following the manufacturer's instructions.

RNAse protection assay. The riboprobes for h*SM22*, m*SM22*, and 18S RNA used for RNAse protection assay (RPA) were labeled with 16-biotin-UTP (Roche Diagnostics). The m*SM22* riboprobe was described before (15). The h*SM22* riboprobe contains the unique 3'UTR of the h*SM22* mRNA and does not cross hybridize with m*SM22* mRNA. Mouse 18S antisense riboprobe was used as an internal control to ensure that the same amount of RNA was used in each RPA reaction. Adult tissues from liver, bladder, esophagus, stomach, aorta, heart, skeletal muscle, and uterus were collected for total RNA isolation using TRIzol reagent (Life Technologies). Ten micrograms of total RNA were used for hybridization with the probes overnight at 42°C, followed by RNase A or T1 digestion at 37°C. After electrophoresis in 5% denatured acrylamide gel, samples were transferred to positively charged nylon membrane. Biotin signals were detected using CDPStar kit following the manufacturer's instructions (Ambion).

RESULTS

Transcriptional activities of hSM22-445 and hSM22-445-intron I promoters in vitro. To determine whether the cloned h*SM22* promoters were transcriptionally active, both h*SM22*-445 and h*SM22*-445-intron I sequences were linked with the lacZ reporter and transfected into PAC1 and A7r5 cells, which are derived from pulmonary and thoracic arteries, respectively (Fig. 1*A*). The h*SM22*-445 promoters showed a high level of activity in PAC1 and A7r5 cells. However, the addition of the intron I sequence reduced the promoter activities by 60% in PAC1 cells and 82% in A7r5 cells (Fig. 1, *B* and *C*). This result suggested that the intron I sequence exerted an inhibitory effect on the h*SM22*-445 promoter in cultured SMC cell lines.

Characterization of transgenic mice harboring hSM22-445/lacZ transgene. To determine the temporospatial expression patterns of the h*SM22*-445 promoter in vivo, we generated three independent transgenic mouse lines carrying the h*SM22*-445/lacZ reporter. The transgene expression patterns in arterial SMCs were similar to that of the m*SM22* promoter (16). The representative expression patterns were shown in Fig. 2. There was no significant transgene expression detected at *embryonic* (*E*) *day 8.5* (*E8.5*) and *E9.5* (data not shown). At *E10.5*, lacZ expression was detected in the bulbus cordis, truncus arteriosus, aortic arch arteries, dorsal aorta, and somites (Fig. 2*A*). The expression continued to increase in the aortic arch, dorsal aorta, common carotid arteries, the outflow tract, and bulbus cordis of the heart. At *E16.5*, lacZ staining was markedly observed in all major arteries in the head and trunk, and the expression in intercostal arteries was apparent (Fig. 2*B*). The transgene expression in the vasculature increased continuously throughout embryogenesis. In the newborn, the expression was clearly seen in aortic arch, carotid arteries, pulmonary trunk arteries, and femoral arteries (Fig. 2*C*). However, starting from the newborn, the expression in the arterial SMCs decreased first in intercostals arteries (Fig. 2*C*). The expression in large arteries such as dorsal aorta began to diminish at ~2 wk after birth. The expression in the whole vasculature was diminished from distal to proximal arteries connected to the heart at ~4 wk (the timing varies between lines) (Fig. 2*D*). Such a downregulation in its expression was never reactivated in the full-grown adult mice (up to 1 year and 10 mo of age) (data not shown). The downregulation of the transgene expression was observed in all three independent transgenic lines, suggesting that it lacked certain regulatory mechanisms in the promoter to maintain the expression in the adult SMCs.

The expression patterns in the heart varied among the different lines. In two independent lines, the transgene expression marked the majority of the right ventricle and diminished at *E15.5*. However, the expression marked both ventricles of the heart and lasted throughout the newborn stage in the third line (data not shown). This observation suggested that the regulation for h*SM22*-445 promoter in the heart was delicate and could be easily influenced by the surrounding sequences at the integration sites in the genome.

Just as in previously characterized m*SM22* promoter in transgenic mice (11, 16, 26), there was no detectable

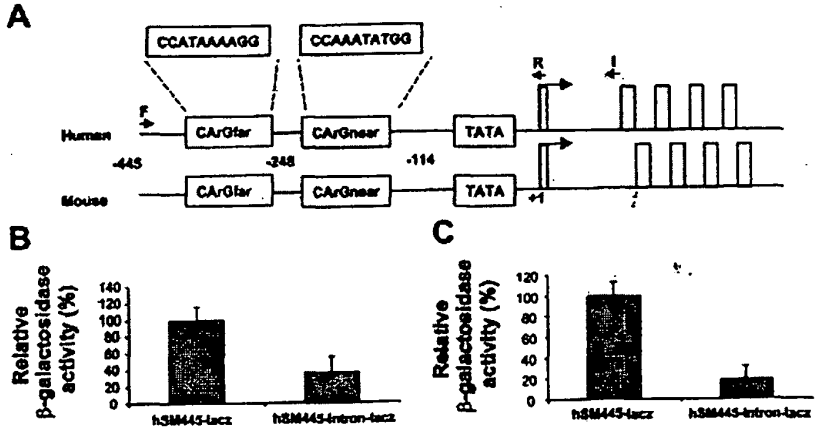

Fig. 1. Gene organization of mouse *SM22α* (m*SM22α*) and human *SM22α* (h*SM22α*) and the human *SM22*–445 (hSM445) and h*SM22*-445-intron (I) promoter activities in smooth muscle cells (SMCs). *A*: *SM22α* gene consists of 5 exons and 4 introns. The 445 bp at the 5' upstream sequence [containing the $CC(AT)_6GG$ (CArG)far and CArGnear boxes] of the *SM22α* gene are highly conserved between mouse and human. Both h*SM22*-445/lacZ and h*SM22*-445-intron I/lacZ were transfected in pulmonary arterial cells (PAC1) (*B*) and A7r5 cells (*C*). h*SM22*-445 promoter activity was designated as 100%. F, forward primer; R, reverse primer; I, intronal primer.

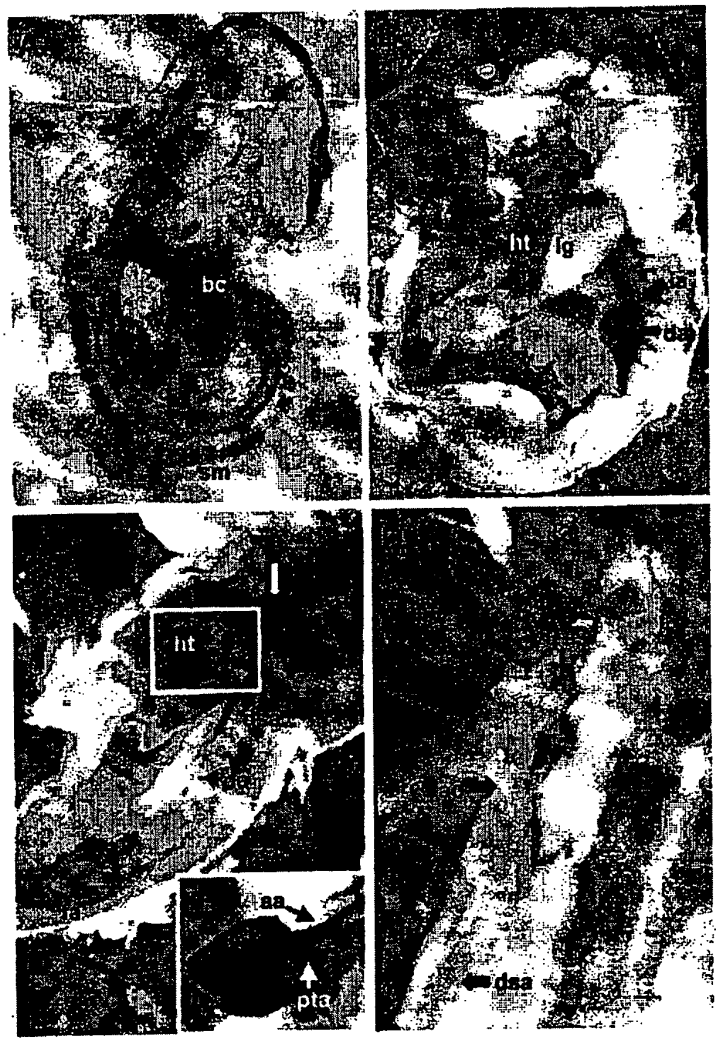

Fig. 2. Expression patterns of the h*SM22*-445/lacZ in transgenic mice. *A*: at *embryonic* (*E*) *day 10.5* (*E10.5*), the lacZ activity was apparent in the bulbus cordis (bc), aortic arch (aa), dorsal aorta (da), and somites (sm) at *E10.5*. *B*: at *E16.5*, expression in the somites diminished. Expression was apparent in the heart (ht), aorta (ao), descending aorta (da), the intercostal arteries (ia) and umbilical arteries (ua). *C*: in newborn mice, lacZ activity was observed in the heart (ht), carotid artery (ca), ascending aorta (aa), pulmonary trunk aorta (pta), descending aorta (dsa), and femoral artery (fa), whereas expression in the dorsal aorta (da) decreased. *D*: LacZ activity in the vasculature was diminished from the cardiovascular system in the adult.

h*SM22* promoter activity in visceral and venous SMCs such as the stomach, the gut, the bronchi, and vena cava at *E16.5* (Fig. 2*B*). The absence of transgene expression in visceral and venous SMCs was obvious throughout development.

Characterization of transgenic mice harboring hSM22-445-intron I/lacZ. Extensive studies have shown that SMC-specific genes, including SM-MHC, SMα-actin, calponin, and *SM22α* share common features in gene organization and CArG box-mediated regulatory network (23). Noticeably, the large intron I in SM-MHC, SM α-actin and calponin genes contains essential regulatory elements for its expression in SMCs (19, 22, 23). Because there are four putative CArG boxes in the intron I sequence of *hSM22*, we were interested in determining whether the intron I of the h*SM22* gene contained any regulatory elements for *SM22α* expression. From 15 independent transgenic mouse lines carrying the h*SM22*-445-intron I/lacZ transgene, we randomly chose five males to characterize their expression at fetal and adult stages. The expression patterns of a representative line were presented here. Staining for lacZ activity was first detected in the bulbus cordis, truncus arteriosus and the somites at *E9.0* (data not shown). At *E10.5*, the expression was apparent in the bulbus cordis, aortic arch arteries, dorsal aorta, and somites (Fig. 3*A*). At *E14.5*, the expression increased in all major arteries including dorsal aorta, carotid artery, and umbilical artery. The expression in intercostal arteries was apparent (Fig. 3*B*). However, the expression in the pulmonary trunk aorta (PTA) was much weaker than in the neighboring aorta at fetal stages, and was absent at newborn stage (Fig. 3, *C* and *D*). These results suggest a repressive effect of the first intron on the promoter activity in PTA SMCs. The repressed transgene expression in the PTA was consis-

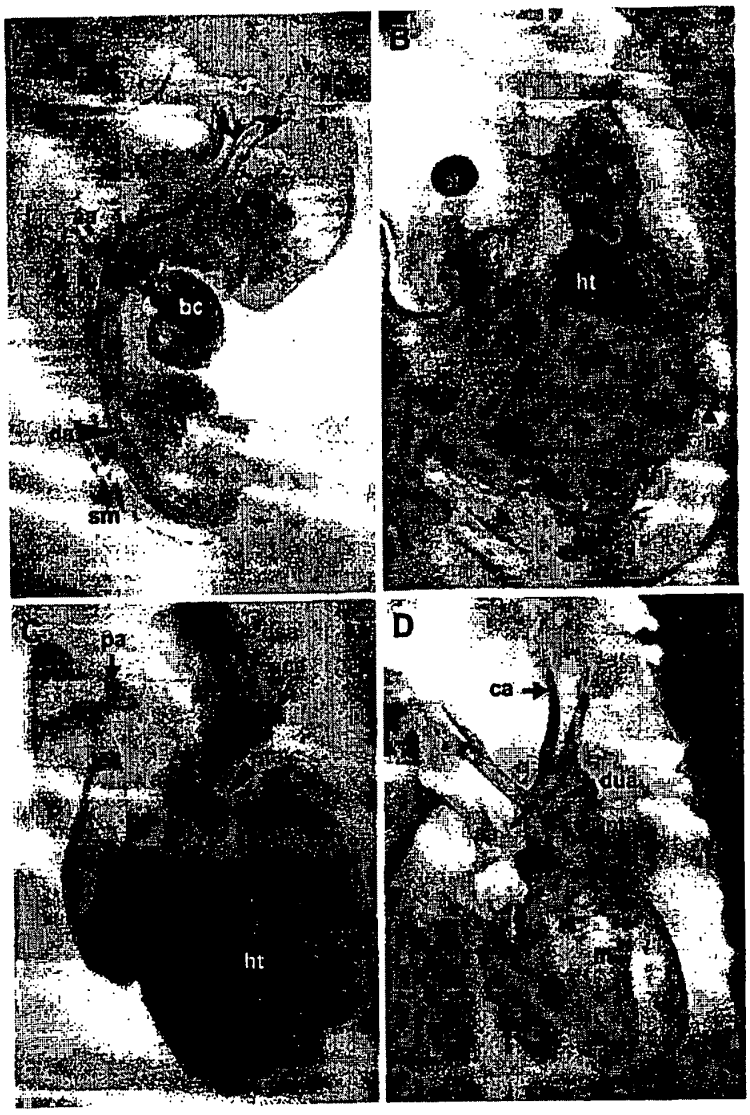

Fig. 3. Expression of h*SM22*-445-intron I/lacZ during development. *A*: at *E10.5*, LacZ activity was detected in bulbus cordis (bc), aortic arches (aa), dorsal aorta (da), and somites (sm). *B*: at *E14.5*, expression was high in the heart (ht), descending aorta (dsa), pulmonary arteries (pa), carotid arteries (ca), umbilical arteries (ua), and intercostal artery (ia). Expression in the somites had decreased to a barely detectable level. *C*: the embryonic heart region at *E14.5* under higher magnification showed that there was much weaker lacZ expression in the pulmonary trunk aorta (pta) than in the ascending aorta (aa). *D*: at the newborn stage, expression in the vasculature significantly decreased and the expression in the pulmonary trunk was undetectable. Note the expression in ductus arteriosus (dua) was apparent.

tently observed in the last kept three independent transgenic mouse lines that showed strong transgene expression. We did not keep the other two lines that showed overall weak transgene expression. The expression in the heart showed certain variation between different transgenic lines (either in the whole heart or in the right ventricle). At the newborn stage, the expression in the heart and the vasculature decreased significantly (Fig. 3*D*). The expression of h*SM22*-445-intron I/lacZ in the vasculature and the heart disappeared at ~4 wk after birth (data not shown). We first observed the fading of expression in intercostals vessels and in dorsal aorta, then in femoral arteries, and last in ascending aorta.

Similar to the h*SM22*-445/lacZ, h*SM22*-445-intron I/lacZ was silent in visceral and venous SMCs throughout development. There was no detectable expression in SMCs of the stomach, the hindgut, the bronchi and vena cava at *E14.5* (Fig. 3*B*) and in adult (data not shown). This study indicated that the h*SM22*-445-intron I promoter showed a temporospatial expression pattern similar to that of the h*SM22*-445 promoter (except in the PTA) and that it lacks the regulatory elements for controlling *SM22α* expression in visceral and venous SMCs in adult mice.

Physical map of BAC harboring hSM22 locus. Recent studies (8) have shown that BAC scanning transgenesis is a powerful tool to identify the regulatory elements for gene expression in vivo. To take advantage of BAC transgenesis in determining the regulatory mechanisms for *SM22α* gene expression in different subtypes of SMCs, we isolated an h*SM22* BAC containing

a 150-kb genomic DNA fragment. This BAC contained the entire h*SM22* gene with ~100 kb of flanking sequence at the 5′ end and 34.24 kb of flanking sequence at the 3′ end (Fig. 4). Sequence analysis using the databases of Celera and GenBank showed that the CGI-40 gene (NM_015996) is present at the 5′ end of the h*SM22* gene with a 2-kb intergenetic sequence and that the PCSK7 gene (NM_004716) is present at the 3′ end with a 0.5-kb intergenetic sequence. The CGI-40 gene is transcribed on the same strand as the *SM22α* gene, 18.470 kb away from the transcriptional initiation site of h*SM22*, whereas human PCSK7 is transcribed on the opposite strand, 32.362 kb away from the transcriptional initiation site of h*SM22* (Fig. 4). It remains to be determined whether the linked genes in this cluster (*SM22α*, CGI-40, and PCSK7) share any common regulatory elements for their transcription.

Temporospatial expression of hSM22 BAC transgenic mice during embryogenesis. To determine the expression patterns of the BAC clone during development, we generated a h*SM22* BAC transgenic mouse. To evaluate the expression of the h*SM22α* transgene during embryogenesis, we performed in situ hybridization on tissue sections at *E13.5* when the expression of endogenous m*SM22α* is high in vascular and visceral SMCs (15). The expression patterns of the h*SM22* transgene were similar to that of the endogenous m*SM22α* gene (Fig. 5). At *E13.5*, h*SM22* gene expression was apparent in all SMCs in the transgenic mouse, whereas no expression was detected in the transgenic-negative mouse (Fig. 5, *A* and *B*). The h*SM22* transcripts were highly expressed in SMCs of the dorsal aorta, tail artery, and iliac artery at *E13.5* (Fig. 5, *C–E*). The expression was also apparent in venous SMCs as seen in the iliac vein (Fig. 5*E*). In addition to the expression in vasculature, the *SM22α* transgene was highly expressed in visceral SMCs, including the esophagus, stomach, intestine, and bronchi of the lungs (Fig. 5, *F–H*). Under high magnification, the expression was easily observed in intercostal vessels (data not shown). These results indicate that the h*SM22α* transgene resembled the temporospatial expression patterns of the endogenous m*SM22α* gene during embryogenesis.

Specific expression of hSM22α BAC transgene in adult. To evaluate the tissue-specific expression of h*SM22α* BAC in adult BAC transgenic mouse, we performed RPA using RNA from different adult tissues. As shown in Fig. 6*A*, the h*SM22α* transgene was specifically expressed in SMC-enriched tissues, including the bladder, esophagus, stomach, and aorta. However, no protected signals were detected in the liver or skeletal muscle. A very weak signal was detected in the heart, possibly from the heart vessels. Therefore, the expression of the h*SM22α* transgene in the BAC transgenic mouse was similar to that of endogenous m*SM22α* in the adult, demonstrating that the human BAC contains the regulatory elements required for *SM22α* expression in both vascular and visceral SMCs during embryogenesis and adulthood.

To further characterize the expression patterns of the h*SM22* transgene expression in adult BAC transgenic mouse, we performed in situ hybridization assay on sections from the heart and uterus. The results showed that the h*SM22* mRNA was detected in the SMCs in PTA as well as in the aorta and the coronary vessels (Fig. 6*B*). A high level of expression of the transgene was also observed in the myometrium layer of the uterus (Fig. 6*C*).

The absence of lacZ expression in the pulmonary trunk region of the h*SM22*-445-intron I/lacZ mice combined with the presence of h*SM22* transgene expression in the pulmonary trunk region in BAC transgenic mice suggests that the inhibitory effect of intron I on the expression of *SM22α* in PTA could be overcome by sequences outside the intron I region in the *SM22α* BAC. These studies point to the existence of a complicated regulatory network for the expression of *SM22α* in different subtypes of SMCs during development.

Overexpression of hSM22 transgene did not affect expression of endogenous SM22α gene in SM22α BAC transgenic mouse. To determine whether the ectopic expression of h*SM22* affected the expression of endogenous *SM22α* gene, we examined the expression level of both h*SM22α* and m*SM22α* in tissues from the h*SM22* BAC transgenic mouse and wild-type littermates. As shown in Fig. 7, h*SM22* mRNA was detected only in the bladder and stomach from the BAC transgenic mouse, whereas the endogenous m*SM22* mRNA showed similar levels of expression from both wild-type and BAC transgenic mice, suggesting that the regulation of the *SM22α* genes was not controlled by the overall level of *SM22α* transcripts.

Taken together, the results of the characterization of h*SM22* BAC transgenic mice at the fetal and adult stages demonstrated that this BAC contained essential regulatory sequences for *SM22α* expression in major arterial, venous, and visceral SMCs. These studies provide a starting point for future characterization aimed at uncovering the regulatory elements for *SM22α* gene expression in different subtypes of SMCs.

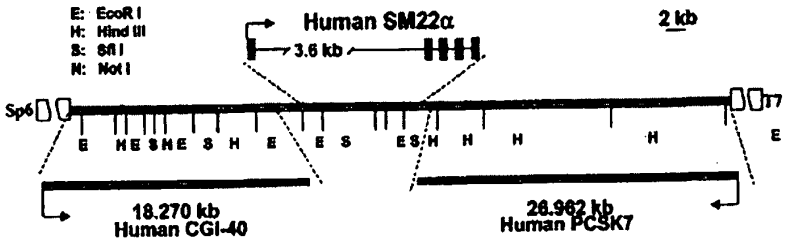

Fig. 4. Physical map of the h*SM22α* bacterial artificial chromosome (BAC). The h*SM22* locus is flanked by CGI-40 and PCSK7 genes. Human CGI-40 is transcribed on the same strand 18.470 kb away from the transcriptional initiation site of h*SM22*, whereas human PCSK7 is transcribed on the opposite strand 32.362 kb away from the transcriptional initiation site of h*SM22*.

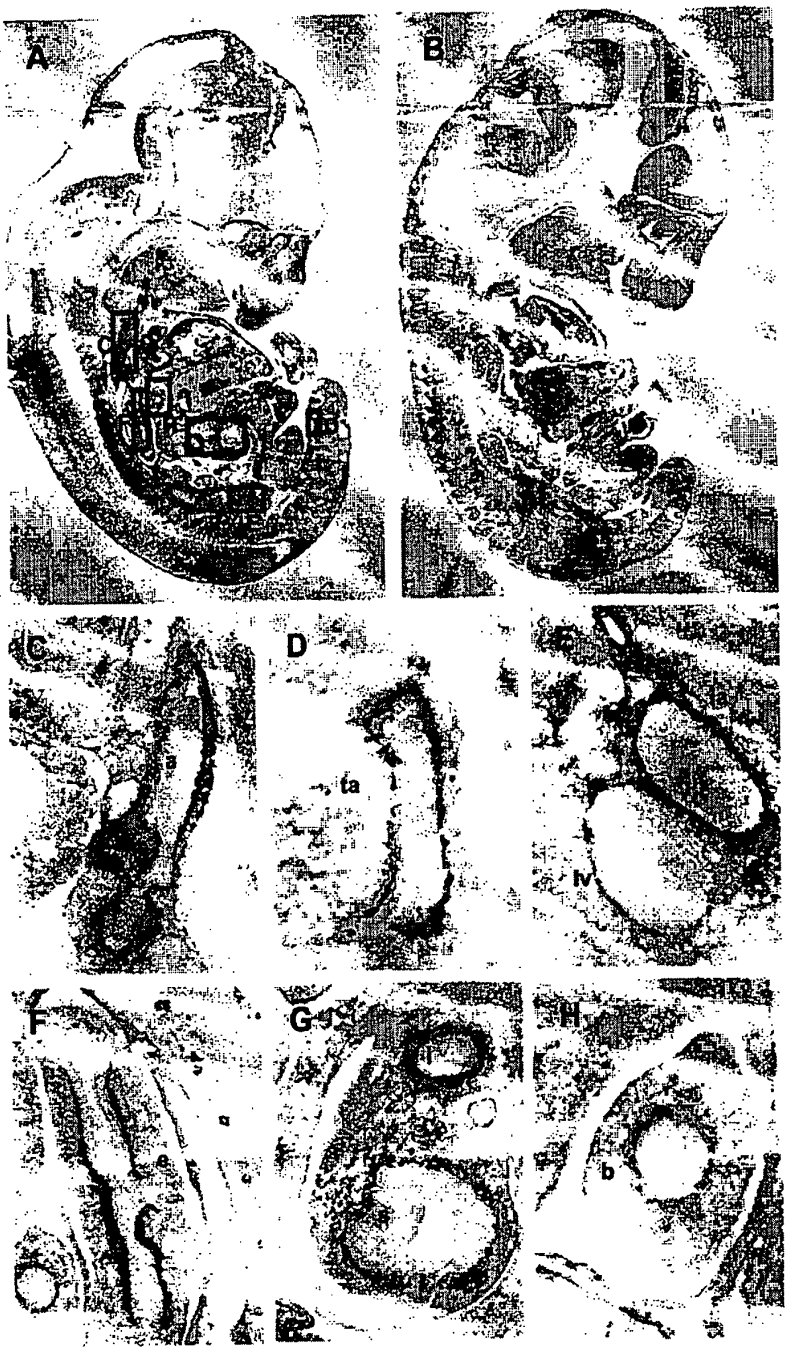

Fig. 5. Expression of the h*SM22α* transgene in a BAC transgenic mouse by in situ hybridization analyses. The red-purple signals correspond to the expression of the h*SM22* transcripts. *A*: at *E13.5*, h*SM22* was expressed in all SMCs, including arterial, venous, and visceral SMCs. *B*: no signal was detected in the transgenic-negative mouse. At high magnification, the transgene expression was apparent in SMCs of the aorta (a) (*C*), tail artery (ta) (*D*), iliac artery (ia) and vein (iv) (*E*), esophagus (e) (*F*), stomach (s) and intestine (i) (*G*), and bronchiole (b) (*H*). The magnification is 20-fold in *A* and *B* and 250-fold in *C–H*.

DISCUSSION

In this report we have demonstrated that the evolutionarily conserved 445 bp 5′ upstream DNA sequence of *SM22α* gene directs transgene expression in arterial, but not in venous nor in visceral SMCs during embryogenesis. However, this promoter activity gradually decreases after birth. To search for additional regulatory elements directing its temporospatial expression in visceral, venous, and adult SMCs, we examined the role of its intron I and a 150-kb h*SM22* BAC in transgenic mice. The results showed that the inclusion of the h*SM22* intron I in the promoter spe-

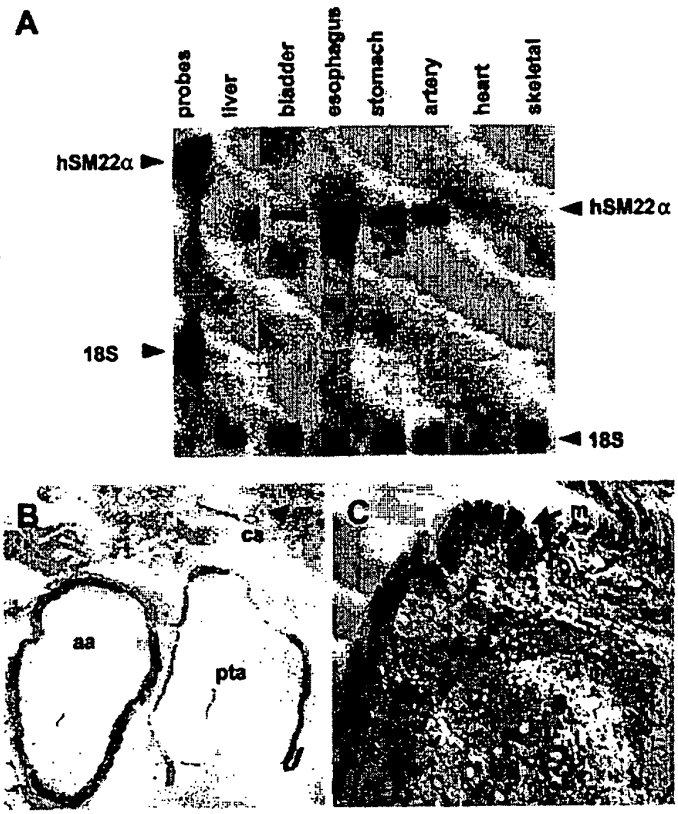

Fig. 6. H*SM22α* transgene expression in the adult BAC transgenic mouse. *A*: biotin-UTP-labeled h*SM22* antisense and 18S antisense riboprobes were used in RNAse protection assay (RPA) to examine the amount of h*SM22* and 18S ribosomal RNA transcripts. Expression of the h*SM22* transgene was observed in all SMC tissues, including the bladder, esophagus, stomach, and aorta. There was no expression in the liver or skeletal muscles; a weak expression in the heart was detected. 18S RNA was used as an internal control to ensure that an equal amount of RNA was used in each RPA reaction. By using the digoxigenin (DIG)-labeled antisense probe for h*SM22* in situ hybridization, h*SM22α* was expressed in coronary artery (ca), aortic artery (aa) and pulmonary trunk aorta (pta) (*B*) and the myometrium (m) layer of the uterus (*C*) in adult BAC transgenic mouse.

cifically suppresses the expression in the PTA-SMCs and that such an inhibitory effect can be overcome by the sequences in other regions of the *SM22α* gene. This report provides direct evidence that additional regulatory sequences are required for controlling *SM22α* gene expression in all types of SMCs during all stages of development.

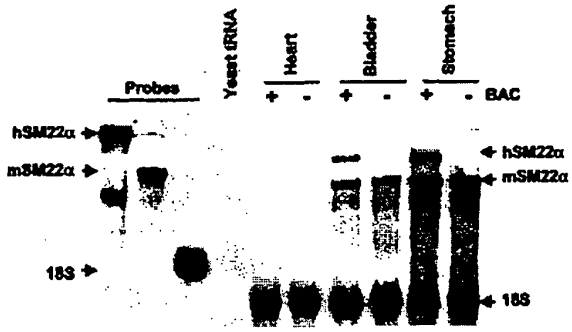

Fig. 7. The effect of overexpressing human *SM22α* transgene on endogenous mouse *SM22α* expression in the BAC transgenic mouse. RPA was used to determine the expression levels of h*SM22* transgene and endogenous m*SM22* in adult smooth muscle tissues from both wild-type mice (−) and BAC transgenic mice (+). The biotin-UTP-labeled h*SM22*, m*SM22* and 18S antisense riboprobes were used in each RPA reaction. The h*SM22* transgene was only expressed in the BAC transgenic mouse. The endogenous m*SM22* was expressed at similar levels in adult SMC tissues from both wild-type and transgenic mice.

Transcriptional regulation of SM22α is controlled by multiple positive and negative modules that differ between different subtypes of SMCs. SMC is known to be highly heterogenous in embryonic origins and pharmacological responses. However, only recently, the heterogeneity of different subtypes of SMCs has been shown at the molecular level. When we first observed that the m*SM22* promoter is specifically expressed in arterial SMCs, but not in venous nor in visceral SMCs, we proposed the notion that a SMC gene can utilize different regulatory mechanisms to control its expression in different subtypes of SMCs (16). This observation was confirmed by two other groups independently (11). Recently, gene subtraction and microarray assay have also identified an array of genes differentially expressed in arteries and veins, which account for their heterogeneity of arteries and veins (1). Extensive evidence further supporting the complexity of SMC gene regulation in different vascular beds is provided by those studies that delineate the regulatory network of SM-MHC, SM actin, and Calponin genes in vivo (23). Recently, the 5-kb CRP1/Csrp1 promoter is also shown to be specifically expressed in arterial but not in venous nor in visceral SMCs in transgenic mice (18). The novel

finding obtained from the present results is that the regulatory mechanism for *SM22α* gene expression in the PTA SMCs is different from that of the rest of the major aortic arteries. Embryonically, the PTA and ascending aorta are originated from the common outflow tract that is divided by the aortico-pulmonary spiral septum and eventually remodeled into two distinct channels (10). The present studies provide molecular evidence that PTA and ascending aorta are distinct in *SM22α* gene regulation.

Perhaps because of the large size of the *SM22*-445-intron I promoter, all of the resulting transgenic mouse lines show consistent and restricted expression in arterial vasculature. This is in contrast to those transgenic mouse lines harboring the h*SM22*-445 promoter, which show occasional ectopic expression in the scalp and skeletal muscles.

Downregulation of the hSM22 promoter in adult SMCs. Although the h*SM22*-445 and the h*SM22*-445-intron I promoters were shown to be transcriptionally active in arterial SMCs during embryogenesis, all of them were downregulated during postnatal development. Such downregulation appeared to follow a pattern first from intercostal arteries, then dorsal aorta, femoral arteries, and, last, ascending aorta. We also observed similar patterns of downregulation in adult SMCs in all m*SM22* promoters containing the 445-bp 5′ upstream sequence (L. Yang and L. Li, unpublished observations). For the *SM22* promoters containing 2.7-kb 5′ upstream sequence with or without the 1.09-kb intronal sequences, the transgene expression patterns in different transgenic mouse lines showed consistent temporospatial expression during embryogenesis. However, the transgene expression in adult arterial SMCs varied among independent lines (Refs. 9 and 16, and personal communication with Drs. M. Husain and D. Dichek). These results suggest that the regulatory network controlling *SM22α* gene expression in adult arterial SMCs is different from that in fetal aortic SMCs.

There are several potential mechanisms for the downregulation of the *SM22* promoter in the adult. The first and most likely is the lack of the regulatory elements for *SM22α* expression in the adult. The likelihood of this mechanism is supported by the *SM22α* BAC transgenic mouse study in this report. This *SM22α* BAC contains essential regulatory elements for *SM22α* expression in major arterial, venous, and visceral SMCs at fetal and adult stages. A second possible mechanism is that the *SM22* promoter can be downregulated in proliferating SMCs in a mouse restenosis model (27). However, this mechanism is less likely to account for the downregulation of the h*SM22*-445 promoter in rapidly growing transgenic mice. We never detected the reactivation of the h*SM22* promoters in full-grown adult transgenic mice. The third possible mechanism is the dominant negative effect of lacZ sequences on the *SM22* promoter. Although it was reported that insertion of the lacZ transgene in the housekeeping gene H-2K results in the methylation of the promoter (4), such a dominant negative effect of lacZ transgene on the *SM22* promoter is less likely. When LacZ transgene was knocked into the intron I (M. Yang and L. Li, unpublished data) or fused in frame to the *SM22α* open reading frame (30), the expression of lacZ is detected in the vascular and visceral SMCs in adult.

Analysis of hSM22α BAC clone in transgenic mice demonstrated that additional regulatory sequences are required for *SM22α*-specific expression in visceral, venous, and adult SMCs. The newly developed BAC scanning transgenic strategy has been shown to be a powerful tool in delineating the regulatory elements for endogenous gene expression in vivo. It has many advantages over the traditional promoter batching (8). The advantage of using BAC DNA for transgenic mice is that the large BAC DNA fragment is more likely to contain all the regulatory elements. Therefore, BAC transgenic mice are likely to show faithful expression patterns of the endogenous gene because of the capacity of BAC in establishing an independent regulatory domain. Because many regulatory elements are found to be more than 10 kb away, it is possible that distal regulatory elements are required for *SM22* expression in other subtypes of SMCs.

We sought the regulatory elements that control the expression of *SM22α* in all types of SMCs. However, no additional regulatory elements were identified in the up to 2.7-kb promoter region of the *SM22α* gene (16, 26). There is speculation that a distinct local chromosome conformation might account for the absence of expression in visceral SMCs (12). To pinpoint the regulatory mechanisms for *SM22α* expression within the full spectrum of development, we analyzed the transgene expression patterns in an h*SM22* BAC transgenic mouse. The expression of the h*SM22* transgene was detected in both vascular and visceral SMCs during embryogenesis and adulthood. We noticed that the h*SM22* transgene expressed at a lower level than the endogenous m*SM22*. Such a difference may reflect the difference in m*SM22α* and h*SM22α* gene expression. Nevertheless, these studies unarguably demonstrate that additional regulatory sequences are needed to control *SM22α* gene expression in all types of SMCs, and that the h*SM22* BAC contains essential regulatory elements required for the expression of the *SM22* gene in major arterial, venous, and visceral SMCs during fetal and adult development.

In conclusion, the characterization of h*SM22* BAC in transgenic mice reported here provides the basis for future analysis of the regulatory network for *SM22* gene expression in other subtypes of SMCs. This work, together with the recently reported characterization of human calponin BAC in transgenic mice (25), shows that the BAC transgenesis approach can help to uncover the regulatory mechanisms for SMC gene expression during development.

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